Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25\textsuperscript{−} into CD25\textsuperscript{+} T regulatory cells

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Indoleamine 2,3-dioxygenase (IDO) is a novel immunosuppressive agent expressed in some subsets of normal and neoplastic cells, including acute myeloid leukemia (AML) cells. Here, we show that IDO expression correlates with increased circulating CD4\textsuperscript{+}CD25\textsuperscript{−}FOXP3\textsuperscript{+} T cells in patients with AML at diagnosis. In vitro, IDO\textsuperscript{+} AML cells increase the number of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells expressing surface CTLA-4 and FOXP3 mRNA, and this effect is completely abrogated by the IDO inhibitor, 1-methyl tryptophan (1-MT).

Introduction

Indoleamine 2,3-dioxygenase (IDO) is a key enzyme in the tryptophan metabolism that catalyzes the initial rate-limiting step of tryptophan degradation along the kynurenine pathway.\textsuperscript{1} Tryptophan starvation by IDO consumption inhibits T-cell activation,\textsuperscript{1,2} while products of tryptophan catabolism, such as kynurenine and O\textsubscript{2}-free radicals, regulate T-cell proliferation and survival.\textsuperscript{1,3} Thus, IDO has been shown to exert an immunosuppressive activity, and cell populations, including regulatory dendritic cells (DCs) and bone marrow (BM)–derived mesenchymal stem cells (MSCs), expressing IDO have the capacity to suppress T-cell responses to auto- and alloantigens.\textsuperscript{3,5}

A wide variety of human solid tumors express IDO.\textsuperscript{6} More recently, we demonstrated that also acute myeloid leukemia (AML) cells, but not their normal counterparts (ie, CD3\textsuperscript{+} hematopoietic stem/progenitor cells [HSCs]), express an active IDO protein, which converts tryptophan into kynurenine and inhibits allogeneic T-cell proliferation.\textsuperscript{7}

Naturally arising CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{+} T regulatory (T\textsubscript{reg}) cells are known to suppress most types of immune response,\textsuperscript{8,9,10} including antitumor immunity.\textsuperscript{10-13} IDO is expressed and is functionally active in placenta, which, in turn, is infiltrated by CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{reg} cells.\textsuperscript{14,16} Moreover, Candida albicans infection increases the number of T\textsubscript{reg} cells because of IDO induction in host antigen-presenting cells (APCs).\textsuperscript{17} In human cancers, tumor-draining lymph nodes contain IDO-expressing DCs that enhance T\textsubscript{reg} cell function.\textsuperscript{18} These data suggest the close relationship between IDO activity and the occurrence of T\textsubscript{reg} cells,\textsuperscript{19} but the mechanism governing the generation of T\textsubscript{reg} cells by IDO-expressing tumors is presently unknown.

In the present study, we investigated whether the expression of IDO by AML cells may play a direct role in the development of T\textsubscript{reg} cells.

Materials and methods

Cells

All human samples were obtained after informed consent was signed, according to institutional guidelines. Approval was obtained from Bologna Hospital Ethical Committee. Buffy coats were obtained from healthy adults during the preparation of transfusion products. BM and/or peripheral blood (PB) samples including at least 70% leukemic cells were harvested from 76 patients with AML at diagnosis. CD3\textsuperscript{+} and CD4\textsuperscript{+} cells were purified from the mononuclear cell (MNC) fraction by MiniMacs high-gradient magnetic separation column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions (purity of CD3\textsuperscript{+} and CD4\textsuperscript{+} cell populations was always greater than 95%). MSCs were generated from BM cells as previously reported.\textsuperscript{20} Murine A20 and CT26 cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) or MEM (Whittaker Bioproducts) supplemented with 10% FCS (Sera Lab, Crawley Down, United Kingdom).
Kingdom), penicillin/streptomycin (50 U/mL), L-glutamine (2 mM), HEPES buffer (Whittaker Bioproducts), and nonessential amino acids (Whittaker Bioproducts), hereafter referred to as complete medium.

RT-PCR
Polymerase chain reaction (PCR) was performed on cDNA as described elsewhere.21 Human INDO: forward 5'-ATGTGGGGCCGAAAGGT-CATTG-3', reverse 5'-AAGTGACTCCGTCTTGACATTGC-3'; mouse INDO: forward 5'-GTCGCTGTATAGGGGCTGT-3', reverse 5'-GATTTAGGAGGTCTTCGAGACTG-3'; and mouse Foxp3: forward 5'-GACCTTACAGCCACTCCT-3', reverse 5'-CTTCTTCTCTCACG-CAACCA-3'. As internal control, human β₂-microglobulin (β2M) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were amplified. PCR products were separated and visualized on 2% agarose gel stained with ethidium bromide.

IDO expression and activity
Human primary AML cells as well as murine cell lines were tested for IDO expression both at the mRNA and protein levels. PCR analysis of human IDO was performed as described. For detection of IDO protein, mouse anti-IDO monoclonal antibody (mAb) (clone 10.1; Chemicon, Temecula, CA) and mouse JgG isotype antibody (Pharmingen, San Diego, CA) were used. Immunocytochemistry analysis was performed on cytospins, as previously reported.22 For IDO activity, the amount of L-tryptophan and L-kynurenine in culture supernatant was measured by high-performance liquid chromatography (HPLC) using a reverse-phase column as previously reported with modifications.4 Cells (10 x 10⁶/well) were cultured in complete medium, and supernatants were collected after 72 hours. After adding N-acetyl-tryptophan and 3-nitro-L-tyrosine (50 μM final concentration), as internal standard for L-tryptophan and L-kynurenine, respectively, 100 μL of supernatant was injected into a C-18 column. The absorbance of column effluent was monitored with the UV detector at a wavelength of 270 nm for tryptophan and 360 nm for kynurenine. The calibration curve between 0 and 12.5 μM was used to quantify the amounts of L-tryptophan and L-kynurenine.

Purification of CD4⁺CD25⁺ and CD4⁺CD25⁻ subsets
CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were isolated by MiniMacs CD4⁺CD25⁺ regulatory T-cell isolation kit (Miltenyi Biotec) according to the manufacturer’s instructions. To achieve highest purity, positive and negative cell fractions were separated over a second column. CD4⁺CD25⁺ cells obtained in the positive fraction were routinely more than 90% of total cells as evaluated by fluorescence-activated cell sorting (FACS) analysis. CD4⁺CD25⁻ T cells accounted for more than 98% of the cells collected in the negative fraction. Purified T cells were used for phenotypic and functional assays. As positive control sample for CD4⁺CD25⁺ T cells, total MNCs were stimulated for 48 hours with mAbs against CD3 and CD28 (Pharmingen, San Diego, CA).

In vitro T-cell culture with AML cells
AML cells (1 x 10⁵/mL) were cultured in RPMI complete medium with 10% allogeneic CD3⁺ T cells/well for 7 days in the presence and absence of optimal concentrations (1000 μM) of the IDO inhibitor 1-methyl-D-tryptophan (1-MT, Sigma-Aldrich, St Louis, MO), as evaluated in preliminary experiments.23 When indicated, total CD3⁺, CD4⁺CD25⁺, and CD4⁺CD25⁻ cells were labeled with CFSE (Molecular Probes, Eugene, OR) by incubation with 2 μM carboxyfluorescin diacetate succinimidyl ester (CFSE) in PBS containing 5% FCS for 5 minutes at 37°C and then used as responders. At the end of culture, cells were collected and used for phenotypic and functional assays. After 24, 48, 72, and 96 hours of culture, T cells were tested for apoptosis by using human Annexin-V and propidium iodide (Bender Medsystems, Burlingame, CA).

Conditioned medium experiments
IDO⁺ and IDO⁻ AML cells (10 x 10⁶/mL) were cultured in complete medium RPMI 1640 (Whittaker Bioproducts) containing 25 μM L-tryptophan in the presence and absence of 1-MT (1000 μM). In selected experiments, L-tryptophan (Sigma-Aldrich) was added to culture medium to a final concentration of 150 μM. After 72 hours, supernatants (conditioned media) were collected and tested by HPLC for L-tryptophan and L-kynurenine concentrations. T cells (10 x 10⁶/mL) were cultured in AML-derived conditioned medium with 10% IDO⁺ AML cells for 7 days. At the end of culture, T cells were collected and used for flow cytometry analysis.

T-cell proliferation and in vitro suppression assays
Standard allogeneic mixed lymphocyte reaction (MLR) was performed as previously described.21 Briefly, naive and leukemic cell-cultured CD3⁺ cells (10⁶/well) were incubated with different numbers of irradiated (3000 Gy) stimulators for 5 days. Then, cells were pulsed with 1 μCi (0.037 MBq) per well of [³H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) and tested as previously described.21 The stimulation index (SI) was calculated for each individual experiment as follows: SI = cpm (counts per minute) (T-cell responders + stimulators)/cpm (T-cell responders).

To test their suppressive activity, control naive CD3⁺ cells and leukemic cell-cultured T-cell subsets were added to cultures consisting of the same donor-derived naive CD3⁺ T cells (5 x 10⁶/well) as responders, and the same number of irradiated allogeneic T-cell depleted MNCs (APCs) as stimulators. After 5 days, cultures were pulsed with 1 μCi (0.037 MBq) per well of [³H]thymidine and tested as previously described.21 Experimental T-cell proliferation was compared with that observed in the presence of control T cells and expressed as a percentage of inhibition.

In vivo experiments
BALB/c mice (8-10 weeks old) were obtained from Charles River (Calco, Italy) and maintained at the Istituto Nazionale Tumori under standard conditions according to institutional guidelines. Female mice were injected intrasplenically with 10⁵ A20 cells or with PBS. Mice received 1-D and L-MT (Sigma-Aldrich) in the drinking water (3.5 mL/day) at the final concentration of 5 mg/mL. After the injection (25 days), mice were killed, and the percentage of CD4⁺CD25⁺ T cells was evaluated in the spleen, pooled lymph nodes, and thymus. Intracellular staining of Foxp3 (FJK-16S) was performed on purified CD4⁺CD25⁺ and CD4⁺CD25⁻ according to the manufacturer’s instructions (e-Bioscience, San Diego, CA).

To test Tregs cell suppressive activity, 5 x 10⁵ CD4⁺CD25⁻ cells were cultured with 5 x 10⁴ accessory cells (ACs) consisting in the whole 3Gy-irradiated spleen, with or without Tregs at the indicated ratio, for 72 hours in complete medium. Anti-CD3 (1 μg/mL; eBioscience) was added to each well for stimulation. [³H] thymidine (1 μCi [0.037 MBq]/well; Amersham Pharmacia Biotech) was added for the last 10 hours of culture and measured in a microplate scintillator counter (Tomtec; Wallac, Turku, Finland). To study in vivo conversion, 10 x 10⁶ Thy1.1-derived CD4⁺CD25⁻ cells, previously labeled with 5 μM CFSE for 15 minutes at 37°C, were transferred by tail-vein injection into recipient mice that had been inoculated intrasplenically 10 days before with 10⁵ A20 cells or subcutaneously with 5 x 10⁵ CT26 cells. The percentage of converted CD4⁺CD25⁺ cells in the spleen was assessed after 10 days. Cells were stained with phycoerythrin (PE)-conjugated anti-CD25, PE-Cy5, anti-CD4, and APC-Thy1.1 antibodies. The percentage of CD25⁺ cells over CFSE⁺ cells was calculated on gated Thy1.1⁺ CD4⁺ cells.

Immunophenotype studies
Dual-color immunofluorescence was performed using the following panel of mAbs: PE- or fluorescein isothiocyanate (FITC)-conjugated human anti-CD3 (clone UCHT1; Pharmingen); PE- or FITC-conjugated human anti-CD4 (clone RPA-T4; Pharmingen); PE- or FITC-conjugated human anti-CD8 (clone HIT8a; Pharmingen); PE-conjugated human anti-CD25 (clone M-A251; Pharmingen); FITC-conjugated human anti–cytotoxic T lymphocyte–associated antigen (anti–CTLA-4, clone CBL, 591F; Cymbus Biotechnology, Hampshire, United Kingdom); FITC-conjugated human anti–HLA-DR (clone L242; Pharmingen); PE- or FITC-conjugated human anti–HLA-DR (clone L242; Pharmingen); PE- or FITC-conjugated human anti–HLA-DR (clone L242; Pharmingen); PE- or FITC-conjugated human anti–HLA-DR (clone L242; Pharmingen); PE- or FITC-conjugated human anti–HLA-DR (clone L242; Pharmingen); PE- or FITC-conjugated human anti–HLA-DR (clone L242; Pharmingen); PE- or FITC-conjugated human anti–HLA-DR (clone L242; Pharmingen).
anti–human CD45RO (clone UCHL-1; Pharmingen); and PE- or FITC-conjugated anti–human CD62L (clone MEL-14; Pharmingen). Negative controls were isotype-matched irrelevant mAbs (Pharmingen). FITC-conjugated anti–mouse CD4 (L3T4), PE-conjugated anti–mouse CD25 (PC61), and the relative isotype controls were all purchased from BD Bioscience (San Diego, CA). Cells were analyzed by using FACSScan equipment (Becton Dickinson). A minimum of 10,000 events was collected in list mode on FACSScan software.

Statistical analysis
Results are expressed as means plus or minus standard deviation (SD). Where indicated, differences were compared using the Student t test and χ-square analysis.

Results
In vivo correlation of IDO expression with increased CD4⁺CD25⁺Foxp3⁺ T cells

Primary AML samples (n = 76) collected at diagnosis were tested for IDO expression. Of these 76 patients with AML, 40 (52%) were positive for both IDO mRNA and protein. Moreover, IDO protein was capable to convert tryptophan into kynurenine and to reduce T-cell alloreactivity (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article).7

To test whether IDO expression in patients with AML correlates, in vivo, with the frequency of Treg cells, PB samples were collected at diagnosis from patients with AML whose circulating T cells were assessable (9 IDO⁺ and 12 IDO⁻) and CD25 expression on gated CD4⁺ cells was evaluated. As shown in Figure 1A, the percentage of CD4⁺CD25⁺ cells was significantly increased in IDO⁺ AML samples compared with IDO⁻ patients or healthy donors (P = .03). The Treg nature of CD4⁺CD25⁺ cells was evaluated by testing the expression of the winged-helix/forkhead transcription factor FOXP3, which is a widely accepted marker for naturally occurring Treg cells. For highly purified CD4⁺ T cells were obtained from the PB of patients with AML and then evaluated for FOXP3 mRNA expression. Figure 1B shows that IDO expression correlates with FOXP3 reverse transcription (RT)–PCR positivity (P = .002). These data reveal a correlation between IDO expression and increased CD4⁺CD25⁺FOXP3⁺ T-cell frequency in patients with AML at diagnosis. IDO-expressing AML cells increase, in vitro, CD4⁺CD25⁺Foxp3⁺ T cells

To investigate the role of IDO expression by AML cells on Treg cell development, we cocultured IDO⁺ and IDO⁻ AML cells with highly purified allogeneic CD3⁺ T cells, obtained from healthy donors, in the presence or absence of the IDO inhibitor 1-MT. The viability of cells cultured in presence of 1-MT was not different from that cultured in medium alone (79% ± 12% and 82% ± 16%, respectively), as well as CD4⁺ and CD8⁺ T-cell frequencies were not modified by the addition of 1-MT (data not shown). Coculture of T cells with IDO-expressing AML cells increased both the percentage of CD4⁺CD25⁺ T cells and the surface expression of CD25 (mean fluorescence intensity [MFI] 150 ± 45 and 980 ± 390 before and after coculture, respectively; Figure 2A-B; P = .001 and P < .001, respectively), whereas coincubation of T cells with IDO⁻ AML cells had no effect on CD25 expression (Figure 2A-B). The addition of 1-MT to cocultures of T cells with IDO⁺ AML cells restored the expression of CD25 to that observed before culture, whereas 1-MT had no effect on T cells cultured with IDO⁻ AML cells (Figure 2A-B). CD4⁺CD25⁺ T cells cultured with IDO-expressing AML cells expressed CTLA-4, which was down-regulated in presence of 1-MT (Figure 2C) as well as HLA-DR, CD62L, and CD45RO (data not shown). The described pattern of surface markers suggests that CD4⁺ T lymphocytes, after coculture with IDO-expressing AML cells, acquire a CD4⁺CD25⁺ Treg cell phenotype, which was markedly inhibited in the presence of 1-MT. This conclusion was corroborated by showing that CD4⁺CD25⁺ but not CD4⁺CD25⁻ cells purified after coculture with IDO-expressing AML cells express FOXP3 mRNA (Figure 2D).

To investigate whether IDO activity in AML cells would result in the generation of a microenvironment that was capable per se to increase CD4⁺CD25⁺ T cells, a conditioned medium obtained from IDO⁺ and IDO⁻ AML cell cultures was used to stimulate naïve CD3⁺ T cells. Moreover, to test whether the availability of an excess of L-tryptophan within the conditioned medium could contrast IDO activity, resulting in a differential induction of CD4⁺CD25⁺ cells, the conditioned medium was generated both with standard (25 μM) and increased (150 μM) starting concentrations of L-tryptophan. According to IDO expression, only the conditioned medium collected from IDO⁺ AML cells had decreased tryptophan and increased kynurenine concentrations (Figure 3A,C). When we compared the contents of conditioned mediums collected from IDO⁺ AML cells previously cultured in 25 μM or 150 μM starting L-tryptophan, the amount of kynurenine
AML cells. Naive CD3+ T cells were stimulated by allogeneic APCs in the presence of AML-cultured or naive autologous T cells. As shown in Figure 4C, T-cell proliferation was markedly reduced when total T cells previously cultured in the absence but not presence of 1-MT were used (P = .02). This effect was markedly increased when AML-cultured CD4+CD25+ T cells were added to cell cultures (P = .03), whereas CD4+CD25+ gave a similar effect to that of cells cultured with 1-MT (Figure 4C). The suppressive activity of CD4+CD25+ T cells obtained after culture with IDO-expressing AML cells was dose dependent (Figure 4D).

Taken together, these data support the hypothesis that CD4+CD25+ T cells induced by IDO-expressing AML cells retain immunosuppressive activity and may be considered bona fide Treg cells.

AML cells convert CD4+CD25- into CD4+CD25+ T cells through an IDO-dependent mechanism

Coculture of IDO-expressing AML cells with CD3+ T cells resulted in the increase of CD4+CD25+ cells, which was paralleled by the decrease of CD4+CD25- cells (Figure 5A; P = .03). This effect could be alternatively explained considering the expansion of CD4+CD25+ cells because of active proliferation, the increased tendency to undergo apoptosis of CD4+CD25- over CD4+CD25+ T cells, or the conversion of CD4+CD25- into CD4+CD25+ T cells.

To address these points, CD4+ T cells were labeled with CFSE before the coculture with AML cells, and then monitored for the dilution of cell-associated fluorescence by flow cytometry. After coculture with AML cells, no significant proliferation was observed for CD25- and CD25+ cells, both in medium alone or supplemented with IL-2 (Figure S2). To assess the tendency of T cells to undergo apoptosis, purified CD4+CD25+ and CD4+CD25- T cells were incubated with IDO+ AML cells and stained with Annexin-V

AML-induced CD4+CD25+ T cells have regulatory activity

T cells obtained after primary coculture with IDO+ AML blasts were compared with autologous naive counterparts for their ability to respond to allogeneic APCs in a secondary MLR. As shown in Figure 4A and 4B, total T cells showed reduced proliferation and IL-2 production (P = .02), which were completely restored by the addition of 1-MT to the same primary coculture. Moreover, when highly purified CD4+CD25+ T cells were used, almost no proliferation and barely detectable IL-2 production were observed (Figure 4A-B). Importantly, CD4+CD25- T cells, which had been completely depleted of CD4+CD25+ cells, showed a marked increase in cell proliferation and IL-2 production compared with that of CD25+ counterparts (P = .001), which was higher than that of naive, unpurified CD3+ T cells. These data point to the functional activity of CD4+CD25+ T cells obtained after culture with IDO-expressing AML cells in reducing T-cell proliferation and IL-2 production.

An additional set of functional experiments was performed to validate the Treg cell nature of the cells induced by IDO-expressing AML cells. Naive CD3+ T cells were capable per se to induce the expansion of CD4+CD25+ T cells expressing AML cells in reducing T-cell proliferation and IL-2 production.

These in vitro data demonstrate that tryptophan catabolism by IDO-expressing AML cells increases CD4+CD25+Foxp3+ T cells.
an in vivo murine system. We found that A20 lymphoma/leukemia
were shown to have increased apoptosis over CD25
presence of converted CD4
AML cells in the presence and absence of 1-MT (1000 μM). Results are representative of 7 independent experiments. *P = .03, experimental versus control sample. (B) Annexin-V staining and flow-cytometry analysis of CD4+CD25+ and CD4+CD25- cells after culture with IDO+ AML cells. Cells were analyzed at different time points. Results are representative of 3 independent experiments. (C) Purity of CD4+CD25- cells. Results are representative of 4 independent experiments. (D) Purified CD4+CD25 T cells were incubated for 7 days with normal MNCs (CTR) or AML cells in the presence and absence of 1-MT. At the end of culture, CD4+ cells were gated and analyzed for the presence of converted CD4+CD25- cells. Results are the mean ± SD of 4 independent experiments. (E) Purified CD4+CD25 T cells were resuspended in the conditioned medium collected from IDO- and IDO+ AML cells and stimulated with IDO- AML cells for 7 days. At the end of culture, CD4+ cells were gated and analyzed for the presence of converted CD4+CD25- cells. Results are representative of 3 independent experiments.

To further explore the capacity of AML-derived conditioned medium and those from transwell cultures to convert CD4+ T cells, we turned to an in vivo murine system. Image was obtained on a Olympus BX-41 microscope (Olympus, Tokyo, Japan) equipped with a 40×0.75 NA objective lens and an Olympus Camera media. No imaging medium or solution was used. Olympus Camera software was used for image acquisition. (B) Functional enzymatic activity. Depletion of tryptophan from the culture medium (expressed as the percentage of the starting concentration in fresh medium) by human MSCs with or without IFN-γ (positive control sample) and A20 cells. Results are representative of 3 independent experiments. (C) BALB/c mice were injected intrasplenically with 10^6 A20 cells (TB indicates tumor-bearing mice), and, from the day of tumor injection, they were treated or not with 1-D and L-MT (NT indicates not treated, n = 21; 1-MT, 1-MT–treated, n = 21). Two groups of mice (naive, n = 15) were not injected with the tumor but received 1-MT. Percentage of CD4+CD25+ T cells among CD4+ T cells in the spleen was assessed by flow cytometry analysis. Averages of data collected from experiments independently performed are reported. The data report the mean ± SD of independent experiments. (D) Intracellular Foxp3 expression in purified CD4+CD25+ T cells obtained from splenocytes of tumor-bearing mice. Results are representative of 4 independent experiments. (E) Anti-CD3-mediated proliferation of naive CD4+CD25- T cells in the presence of increased numbers of CD4+CD25+ cells obtained from splenocytes of tumor-bearing mice (B). As a positive control, CD4+CD25- cells were stimulated in the absence of CD4+CD25+ T cells (C). Proliferation was evaluated after 3 days by thymidine incorporation assay. Results are expressed as cpm and represent the mean ± SD of 4 independent experiments. *P = .03, experimental versus control sample. (F) In vivo conversion experiments. Purified Thy1.1+ CD4+CD25+ T cells (10 x 10^6) were transferred into BALB/c mice bearing A20 or CT26 tumors. After 10 days, spleens were collected and labeled with Thy1.2, CD4, and CD25; Thy1.1+ CD4+ cells were gated and analyzed for CD25 and CFSE expression. Cumulative data of the CFSE+CD25+Thy1.1+ cells conversion in spleens cells are reported and represent the mean ± SD of 4 experiments.
well as reduces the concentration of tryptophan from culture medium (Figure 6B). Thus, we assessed whether intrasplenic injection of A20 cells would increase the frequency of CD4+CD25+ T cells. As shown in Figure 6C, the percentage of CD4+CD25+ T cells was higher than that of non–tumor-bearing mice. Such increase was time dependent and maximal at day 25 after tumor challenge (data not shown) and, more important, was reduced by the treatment of tumor-bearing mice with 1-MT (P = .03). Purified CD4+CD25+ T cells derived from spleens of tumor-bearing mice were both phenotypically and functionally Treg cells, as shown by Foxp3 expression and the ability to suppress anti-CD3–mediated T-cell proliferation, respectively (Figure 6D-E). To investigate whether conversion may be the mechanism of Treg cell expansion in IDO+ tumor-bearing mice, CD4+CD25− T cells, purified from spleens of Thy 1.1 congenic mice, were labeled with CFSE and inoculated into Thy1.2 mice bearing IDO+ A20 tumors. For control tumor, we used the CT26 colon carcinoma, which lacks IDO expression (data not shown), but has been recently shown to expand a population of well-characterized Treg cells by conversion of CD4+CD25− into CD25+ T cells. In both cases a group of mice was treated with 1-D and L-MT, as previously described.6 After about 10 days from tumor challenge, spleens were collected and Thy1.1+ CD4+ donor lymphocytes were analyzed for the expression of CD25 as function of conversion and for CFSE dilution as a function of proliferation. Both A20 and CT26 tumors were capable to induce the conversion of CD4+CD25− into CD4+CD25+ T cells (Figure 6F). However, the treatment with 1-MT was effective in blocking conversion of CD4+CD25− into CD4+CD25+ T cells (P < .05) in A20-bearing mice but not in CT26-bearing mice. Moreover, no significant difference was observed as for CFSE dilution (data not shown), indicating that the expansion of Treg cells induced by IDO-expressing tumor cells was due to conversion of CD25− into CD25+ T cells in the absence of proliferation.

Collectively, these data demonstrate that IDO expression by tumor is directly responsible for in vivo Treg cells expansion by conversion of CD4+CD25− into CD4+CD25+ Foxp3+ T cells.

Discussion

Since its demonstration as a potent immunosuppressive agent, IDO has been widely investigated for the induction of immunologic tolerance.26 Recently, IDO has been shown to be expressed in a wide variety of solid tumors and to prevent T-cell–mediated immunity in mouse tumor models.6 However, little is known about the mechanism(s) by which IDO-expressing tumor cells inhibit antitumor immunity. In this report, we show that IDO, which is constitutively expressed in a significant portion of patients with AML at diagnosis, directly expands CD4+CD25+ Treg cells by the conversion of CD4+CD25− T cells.

Tumor cells, including leukemia cells, are known to create an inhibitory microenvironment for the immune system, which could be counteracted by the optimal securion of immunomodulatory cytokines, such as IL-12.28 Recent investigations have established the role of IDO in inducing tolerance to tumors.6,18 Published data demonstrate that the antitumor effect of IDO blockade was completely dependent on the presence of a fully competent immune system, thus suggesting that IDO acts by deregulating the host immune response. In the present study, we show that in patients with AML IDO expression is associated with an increased number of circulating CD4+CD25+Foxp3+ T cells. Such correlation is physiologic in the placenta, where decidua cells expressing IDO are fully infiltrated by CD4+CD25+ T cells,15 which have a major role in the induction of maternal tolerance against fetal alloantigen.16 Accordingly, our data demonstrate that IDO-expressing leukemia cells expand, in vitro and in vivo, a population of CD4+CD25+ Foxp3+ T cells, which functionally act as bona fide Treg cells.

Different mechanisms have been proposed for IDO-mediated immunoregulation during infection, pregnancy, autoimmunity, transplantation, and neoplasia.1 Local depletion of tryptophan and/or the production of proapoptotic kynurenines are considered responsible for the multiple effects on lymphocyte proliferation and survival after IDO induction.1,2,29 In particular, Th1 but not Th2 clones can rapidly undergo cell death in presence of low concentrations of tryptophan metabolites of the kynurenine pathway, such as 3-hydroxyanthranilic and quinolinic acids.30 In the present study, we were not able to demonstrate any role for IDO in the induction of apoptosis of T-cell subsets. Conversely, we have been able to demonstrate, in vitro, that IDO-expressing AML cells directly increase Treg cell frequencies in a mechanism of conversion from CD4+CD25− T cells. Moreover, although mice experiments cannot definitely rule out the possibility that Treg cell frequencies may be affected in vivo by several different mechanisms, our results suggest that conversion may be an important pathway by which IDO-expressing tumors expand Treg cells.

Originally thought to be of restricted thymic origin, recent evidence indicates that Treg cells can also be generated in the periphery upon subimmunogenic stimuli (ie, in the presence of suboptimal doses of antigen and APC activation),31,32 a situation resembling tumor-host interaction. Accordingly, murine tumors of different histology are capable to expand Treg cells by converting CD4+CD25− into CD25+ T cells.25 Colombo et al25 postulated that tumor-derived soluble factors, such as transforming growth factor (TGF)−β1,34 which are known to regulate the conversion of CD4+CD25− into CD4+CD25+ T cells,35 may play a critical role in inducing the conversion into Treg cells. Although we could not rule out that, in our system, a fraction of converting T cells may be generated in the presence of leukemia-derived production of soluble factors, such as TGF-β1, we found that T-cell conversion was completely abrogated by IDO blockade in AML cells. These data point to a direct role of IDO production in the expansion of converting Treg cells by AML cells and are in agreement with the results by Puccetti et al demonstrating that in a nontumoral mouse model, tryptophan catabolism favors the emergence of CD4+Foxp3+ Treg cells by conversion from CD25−Foxp3− cells.36 It remains to be elucidated how the modulation of tryptophan catabolism by IDO-expressing AML cells may be implicated in the conversion of CD4+CD25− cells into CD4+CD25+ cells. In particular, the accumulation of small molecules within tumor microenvironment has been recently demonstrated to affect significantly tumor-infiltrating cell populations.37 Here we show that the conditioned medium obtained from IDO+ but not IDO− AML cells was capable, per se, to induce the conversion of CD4+CD25− T cells. These data suggest that IDO− AML cells induce a tumor microenvironment containing reduced concentrations of tryptophan and high concentrations of kynurenine, which may have a role in AML-induced expansion of Treg cells by conversion of CD4+CD25− T cells.
In conclusion, IDO production by AML cells directly increase T_{reg} cells through the conversion of CD25$^-$/CD25$^+$ T cells. IDO expression can be regarded as a novel mechanism of leukemia escape from immune control and its inhibition may represent a novel antileukemia therapeutic strategy.

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Authorship

Contribution: A.C. was responsible for design of the research, flow cytometry, human functional tests, statistical analyses, writing of the manuscript, and review of the accuracy of the reported results; G.B., S.R., S.P., A.L., E.F., and V.S., for flow cytometry, functional tests, immunocytochemistry, and ELISAs; M.A., for molecular biology; B.V., for mouse experiments; I.D. and F.F., for HPLC analysis; M.M., A.L.H., and M.B., for critical review of the manuscript; M.P.C., for contribution to manuscript writing; and R.M.L., for contribution to the research plan and manuscript writing.

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Modulation of tryptophan catabolism by human leukemic cells results in the conversion of CD25 <sup>−</sup> into CD25<sup>+</sup> T regulatory cells

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